Université de Sherbrooke

Études *in vivo* de l'épissage alternatif de l'exon EDIIIA du gène de la fibronectine

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ABBREVIATIONS

A	adenosine
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
°C	degree celsius
DEAE	diethylaminoethyl cellulose
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
Fig.	figure
FN	fibronectin
G	guanosine
g	gram
hr	hour
kDa	kilodalton
LB	L-broth
M	molar (moles/litre)

min	minutes
ml	milliliter (10^{-3} L)
mM	millimolar (10^{-3} M)
mmole	millimole
mRNA	messenger RNA
ng	nanogram (10^{-3} gram)
nt	nucleotide
^{32}P	radioactive isotope of phosphorus
PCA	phenol:chloroform:isoamyl alcohol (25:24:1)
poly A	poly adenosine
RNase	ribonuclease
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
T	thymidine
Tris	tris (hydroxy methyl) aminoethane
µCi	microcurries
µg	microgram (10^{-6} gram)
µl	microlitre (10^{-6} L)
µM	micromolar (10^{-6} M)

Résumé

Les évidences que l'exon EDIIIA varie dans son inclusion au niveau de l'ARN messager dans différentes lignées cellulaires suggèrent que l'épissage alternatif de cet exon est régulé. D'autres études ont démontré qu'il y avait une séquence "splicing enhancer (SE)" à l'intérieur de l'exon EDIIIA qui était nécessaire pour son inclusion dans l'ARN messager. Des études effectuées *in vitro* ont identifié une séquence riche en purines de 9 nt qui stimule l'utilisation du site d'épissage 3' en amont de EDIIIA. Cette séquence riche en purines lie les protéines SR. Nous avons construit deux mini-gènes dont l'un contient l'unité d'épissage alternatif EDIIIA et l'autre possède une délétion de la séquence riche en purines. Ces mini-gènes ont été transfectés dans des cellules WI38VA13 et MRC5, deux lignées dans lesquelles le pré-ARNm de la fibronectine est épissé de façon différente. Le patron d'épissage des mini-gènes a été analysé par RT-PCR et a démontré que la séquence riche en purines est nécessaire pour l'inclusion de l'exon EDIIIA dans l'ARN messager *in vivo*. Étant donné que l'élément "splicing enhancer" est requis pour l'inclusion de l'exon EDIIIA dans les deux lignées cellulaires, nos résultats suggèrent que la séquence riche en purines pourrait jouer un rôle dans la régulation de l'épissage alternatif.

Nous avons étudié l'effet de l'expression des protéines SR sur la régulation de l'épissage alternatif de l'exon EDIIIA. Sachant que le niveau d'expression des protéines SRp40, SF2, et SC35 est moins abondant dans les cellules MRC5 que dans les cellules WI38VA13, nous avons tenté de surexprimer ces protéines SR dans les cellules MRC5. La surexpression des protéines SC35 et SF2 dans les cellules MRC5 inhibe la croissance

des cellules. Nous avons obtenu des cellules stables transfectées par le cDNA de SRp40 mais le niveau de protéine SRp40 phosphorylée dans ces cellules était réduit. Cette diminution de la protéine SRp40 phosphorylée a été accompagnée par une réduction dans le niveau de produits incluent l'exon EDIIIA. Ces résultats suggèrent que la séquence riche en purines et la protéine SRp40 jouent un rôle dans la régulation de l'épissage alternatif.

Abstract

Cell-specific differences in the level of inclusion of the fibronectin EDIIIA exon suggest that the alternative splicing of this exon is regulated. Previous studies have revealed that the EDIIIA exon contains a splicing enhancer which stimulates its inclusion into the mRNA. Further investigation identified a 9 nt purine-rich sequence which stimulates the use of the upstream 3' splice site of EDIIIA *in vitro*. This sequence is bound by SR proteins. To study the possible role of the purine-rich sequence in the regulation of alternative splicing of the EDIIIA exon, a mini-gene construct was made containing the EDIIIA alternative splicing unit. Another mini-gene construct carried a deletion of the purine-rich sequence. These mini-genes were transfected into two cell lines (WI38VA13 and MRC5) which splice EDIIIA differently. The splicing pattern of these mini-genes was analyzed by RT-PCR and the results indicate that the purine-rich sequence is necessary for the inclusion of the EDIIIA exon *in vivo*. Because the splicing enhancer was required for EDIIIA inclusion in both cell lines, our results suggest that the purine-rich sequence may play a role in the regulation of alternative splicing.

We also studied the effect of SR protein expression in the regulation of the alternative splicing of this exon. Because the level of SRp40, SC35, and SF2 are reduced in MRC5 cells which skip EDIIIA more often than WI38VA13 cells, we attempted to overexpress these SR proteins in MRC5 cells. Overexpression of SC35 and SF2 in the MRC5 cells was probably toxic and inhibited the growth of the cells. Growing cells were obtained only by stable transfection of the SRp40 cDNA. Surprisingly, the amount of phosphorylated SRp40 protein expressed in the MRC5 cells was reduced. This decrease in phosphorylated SRp40 protein was accompanied by a reduction in the level of inclusion of the EDIIIA exon. These results suggest a role for the purine-rich sequence and SRp40 in the regulation of alternative splicing of the fibronectin EDIII-A exon.

Introduction

Constitutive Splicing

Constitutive splicing is the process by which a precursor mRNA (pre-mRNA) is converted into a mature mRNA through the removal of the introns and joining of the exons in a precise manner. This process takes place in a large ribonucleoproteic complex known as the spliceosome. The complex is made by the sequential binding of several small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6, and U5 to the pre-mRNA along with the interaction of many other non-snRNP particles. The reaction may be seen as a two-step process utilized from yeast to mammals (Green, 1986; Padgett *et al.*, 1986; Guthrie and Patterson, 1988). The first step involves recognition and cleavage of the 5' splice site and formation of a 2'-5'- phosphodiester bond between the 5'- terminal G residue of the intron and an A residue at the branch site. The second step involves cleavage at the 3' splice site and ligation of the two exons with the release of the intron in the form of a lariat.

Sequence analysis of many different pre-mRNAs has revealed four conserved regions involved in the splice site recognition. These regions are the 5' splice site, the 3' splice site, a polypyrimidine track just upstream of the 3' splice site, and the branch site found between 18 and 40 nucleotides upstream of the 3' splice site. These sequences are not 100% conserved in mammalian genes. Each of these regions is involved in the interaction with snRNPs or other splicing factors during the assembly of the splicesome. In mammals, assembly of the spliceosome begins with the interaction of U1 with the 5' splice site, and $U2AF^{65}$ (U2 snRNP auxiliary factor) with the polypyrimidine tract near the 3' splice site (Fig. 1). Then there is binding of U2 snRNP, guided by $U2AF^{65}$, to the branch site allowing for the formation of a bulged out adenosine since this base does not base-pair with the U2 snRNA. This bulged-out adenosine can be used as a nucleophile in the first catalytic reaction (Query *et al.*, 1994). The interactions of U1 and U2 snRNPs

with the 5' splice site and the branch point, respectively, involve base pairing between complementary regions of U1 and U2 snRNAs (Maniatis and Reed, 1987; Green, 1991; Rosbash and Séraphin, 1991; Michaud and Reed, 1991). Once U2 snRNP has bound to the branch site, a triple-snRNP particle made up of U4, U5, and U6 snRNAs enters the complex and completes the basic spliceosome. U4 and U6 snRNAs are base paired to each other but once the triple-snRNP enters the spliceosome there is a disruption of this base pairing and U4 is released. At this point, U6 undergoes base pairing with U2. There is also destabilization of U1 binding of the 5' splice site which allows for U5 and U6 snRNAs to interact with the free 5' splice site region. These events all take place before the first catalytic step. The network of snRNA-snRNA and snRNA to intron interactions leads to the formation of the active site required for the first catalytic step of splicing (Madhani and Guthrie, 1994; Nilsen, 1994; Sun and Manley, 1995). The products of this first step are the intermediate products, an upstream exon and the lariat intron still covalently linked to the downstream exon. The second catalytic step of splicing also requires a major conformational change in the spliceosome (Schwer and Guthrie, 1992) leading to the products: the joined exons and the lariat intron still complexed with the U2, U5, and U6 snRNPs.

Some non-snRNP protein factors involved in splicing have also been identified. Among them are the SR proteins, a family of serine- and arginine-rich nuclear phosphoproteins considered as essential splicing factors (Zahler *et al.*, 1992). They have a possible role in stabilizing the interactions of the snRNPs allowing for spliceosome assembly. These proteins were first classified as a family due to their common epitope recognized by the antibody mAb104. The family consists of at least six members with approximate molecular masses of 20, 30, 40, 55, and 75 kDa. These proteins were found to be conserved between different species from Drosophila to humans. Another member of this family has recently been identified and given the name 9G8 (Cavaloc *et* al., 1994).

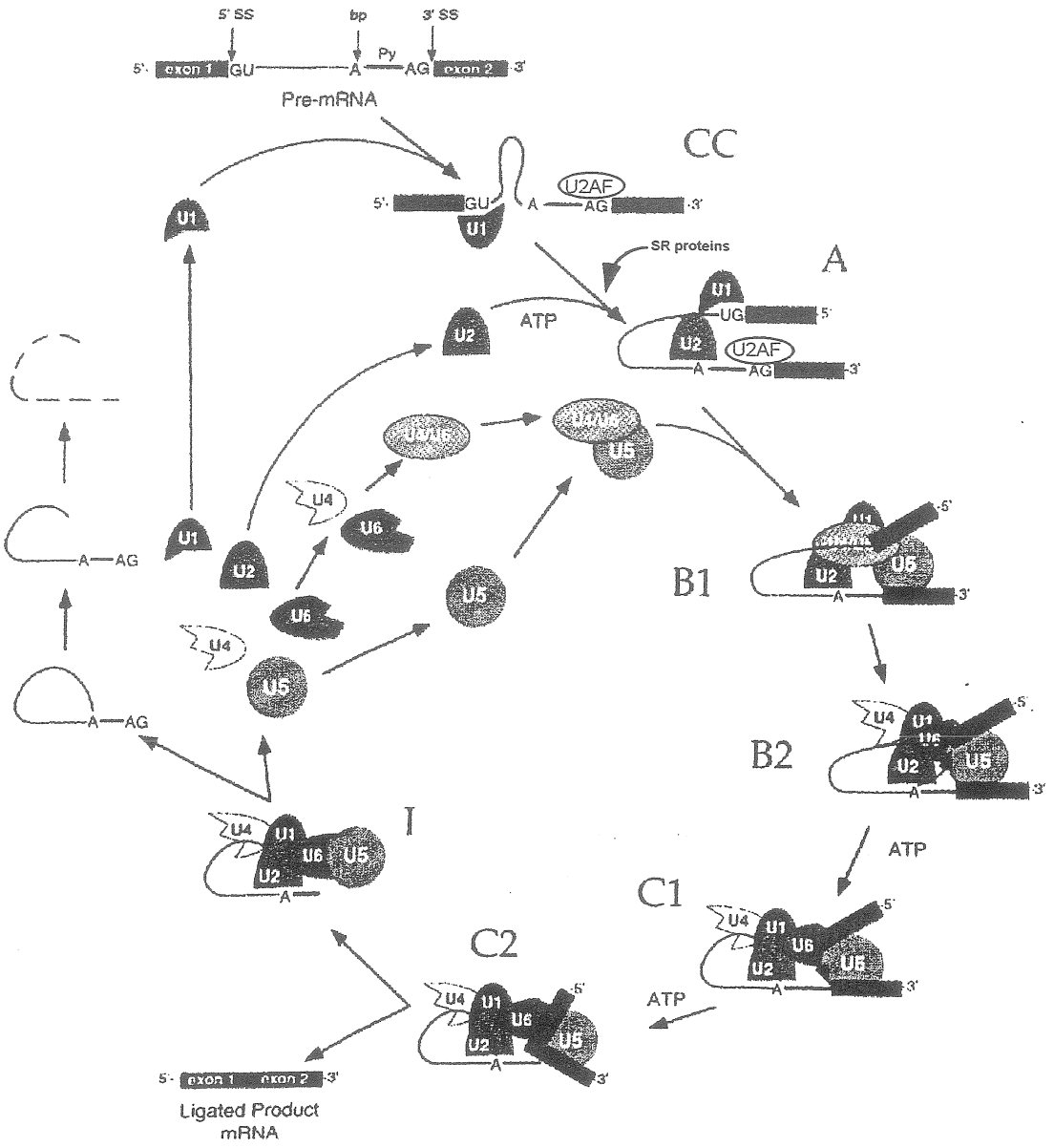


Figure 1: Schematic representation of the involvement of the small nuclear ribonucleoproteins (snRNP) particles and the some non-snRNP factors in pre-mRNA splicing. The pre-mRNA (top line) containing two exons separated by an intron enters the splicing complexes in association with snRNPs and exits as ligated mRNA (bottom line) and the release of a lariat intron (left border). CC, A, B1, B2, C1, C2, and I represent macromolecular complexes within the splicing pathway. 5' SS, 3' SS, bp, and Py indicate 5' and 3' splice sites, branch site, and polypyrimidine tract, respectively. The snRNPs are U1, U2, U4, U5, and U6 and the non-snRNPs factors are U2AF and SR proteins. (Modified from Moore *et al.*, 1993)

The SR proteins all contain at least one RNA recognition motif (RRM) at their N-terminus as well as an arginine/serine-rich (RS) motif at their C-terminus. They have been identified as essential splicing factors because they are able to complement splicing deficient S100 extracts (Krainer and Maniatis, 1985; Krainer *et al.*, 1991; Zahler *et al.*, 1992). SR proteins have been noted to have some distinct features in the splicing of some pre-mRNAs, such as for the splicing of the HIV tat pre-mRNA which is dependent on the presence of SF2 but not SC35 (Fu, 1993), or the ability for SC35 but not SF2/ASF to replace 9G8 in the splicing of nuclear extracts depleted of 9G8 (Cavoloc *et al.*, 1994).

SF2/ASF was found to assist U1 snRNP binding to the 5' splice site through interactions with the U1 70kDa protein. The binding of SF2/ASF to the pre-mRNA may be needed prior to binding of U1 snRNP for complex formation (Kohtz *et al.*, 1994; Jamison *et al.*, 1995). SC35 was also shown to promote commitment complex formation in nuclear extracts (Staknis and Reed, 1994). Another set of studies revealed that the SR proteins SC35 and SF2/ASF could simultaneously interact with the U1 70kDa protein and $U2AF^{35}$ (Fu and Maniatis, 1992). Because $U2AF^{35}$ is associated to $U2AF^{65}$ through another protein interaction, these studies provide a possible role for the SR proteins in bringing the 5' and 3' splice sites closer together during spliceosome assembly. From these results it is evident that the SR proteins are a very important family of proteins with several functions in the splicing reaction.

The conserved sequences mentioned above are very important for the identification of the different splice sites but they are not sufficient to define the splice sites efficiently since there are similar sequences found at other positions in the pre-mRNA that are not used as splice sites. Some of these other sites can be used as cryptic splice sites when mutations to the original splice site compromise its utilization. The presence of the SR proteins appear to have a role to play in helping define the different splice sites. There may be other factors also involved but they have yet to be identified.

Splice Site Selection

How does the splicing apparatus identify splice sites? Much work has been put into answering this question and we are starting to place some portions of the puzzle together. The consensus sequences at the 5' and 3' splice sites as well as those of the branch site do play a important role in the identification of splice sites. However, these sites alone are not able to define the splice sites because some deviate from the consensus sequences and similar sites are found elsewhere in the pre-mRNAs that are never recognized as splice sites unless mutations are produced in the original sites.

Splicing Signals

One parameter which has a great influence on the selection of splice sites is the strength of splice sites. A strong 5' or 3' splice site, one which is used efficiently, generally has a sequence very similar to that of the consensus sequences, and those that deviate from the consensus are usually used less efficiently. Mutational studies of a constitutive exon to reduce the strength of the splice signals rendered the exon alternative; i.e. it was skipped with a higher frequency (Dominski and Kole, 1992). In other cases there was activation of cryptic sites when the original splicing signals were mutated.

The strength of the different sites may thus play an important role in the alternative splicing since the balance between strong and weak sites along with the involvement of the local sequences will push the system to choose the best splice sites

under specific conditions. Examples of 5' splice sites involved in the regulation of alternative splicing are those of exon 4 of the preprotachykinin gene which has a poor complementary to U1 RNA (Kuo *et al.*, 1991), and the weak 5' splice site of the Drosophila P-element flanked by competing sites which lead to a diminished recognition of the authentic site (Siebel and Rio, 1990).

Examples that involve regulation at the 3' splice sites featuring multiple or disrupted branch sites as well as long polypyrimidine tracts have also been found (Gattoni *et al.*, 1988; Goux-Pelletan *et al.*, 1990; Helfman and Ricci, 1989; Norton and Hynes, 1990). Regulator elements have also been found within the polypyrimidine tract such as a negative regulator element found in the rat β-tropomyosin polypyrimidine tract of exon 7 leading to the exclusion of this exon in non-muscle cells (Guo *et al.*, 1991; Helfman *et al.*, 1990). The human α-tropomyosin gene has a poor branch site which influences the exclusion of one exon in non-muscle cells (Graham *et al.*, 1992).

The compatibility between different splice sites has also been shown to be involved in splice site use. In such a case two splice sites are in competition for a single acceptor site but only one of the two sites is used all the time. Examples of this kind have been seen in the myosin light chain 1/3 gene where selection between exons 3 and 4 depends on the upstream constitutive 5' splice site (Periasamy *et al.*, 1984; Strehler *et al.*, 1985). Usually exon 3 is excluded from the mRNA and exon 4 is included, but when the upstream exon is exon 2 there is inclusion of exon 3. No other upstream 5' splice site has shown this behavior for the selection of exon 3 in these constructs. This effect was also noted in the experiments involving the substitution of the E1a 3' splice site and branch-point region with that of the β-globin branch point and 3' splice site. Here, there is a

switch from the predominant splicing to the 13S 5□ splice site to that of the distal (or upstream) 9S 5□ splice site (Ulfendahl *et al.*, 1989).

Exon and Intron Sequences

Other sequences that influence alternative splicing have been found in the exons and introns. These sequences do not make up any part of the consensus sequences of the four main splicing elements and are known as splicing enhancers or repressors. The enhancer and repressor sequences do just that, either enhance or repress the use of the splice sites they act on. Examples of exon sequences involved in stimulating the use of alternative splice sites have been found for the human growth hormone (Hampson *et al.*, 1989), cardiac troponin T (Cooper and Ordahl, 1989), fibronectin (Lavigueur *et al.*, 1993; Mardon *et al.*, 1987), and calcitonin/CGRP (Cote *et al.*, 1992). As for intron sequences some have been found that regulate the selection of an upstream exon such as in chicken β-tropomyosin (Balvary *et al.*, 1992), fibronectin (Huh and Hynes, 1993), and the c-src gene (Black, 1992).

Exon sequences involved in the repression of the use of splice sites have been found in the K-SAM exon of the fibroblast growth factor receptor 2 pre-mRNA (Del Gatto and Breathnach, 1995), the human fibronectin EDIIIA exon (Caputi *et al.*, 1994), and in the human immunodeficiency virus type 1 tat exon (Amendt *et al.*, 1994). An exon sequence near the 3' splice site of a β-tropomyosin alternative exon inhibits the use of the splice site via the formation of a secondary structure (Clouet d'Orval *et* al., 1991; Libri *et* al., 1991).

Studies done on the position of some of these sequences have revealed that their distance from the splice sites is important for their activity. In the case of the human fibronectin splicing enhancer, it does not function when located further than 293 nt downstream of the 3' splice site of exon EDIIIA (Lavigueur *et al.*, 1993). The repressor sequence TAGG of the K-SAM exon looses its repressor effect when the 3' and 5' splice sites are brought closer together (Del Gatto *et al.*, 1996).

Regulation of Alternative Splicing

Alternative splicing involves the same basic two step process as that of constitutive splicing except it allows for a diversity in the mRNAs produced from a common pre-mRNA. This process also allows for the post-transcriptional regulation of many biological molecules. These can involve on/off regulation of the products of particular genes such as in Drosophila sexual differentiation regulated by *Sex lethal* (Bell *et al.*, 1988) and *transformer* (Boggs *et al.*, 1987) regulatory genes, or could lead to alternative products each with a separate function, as is the case for the isoforms of the glycolytic enzyme pyruvate kinase gene (Noguchi *et al.*, 1986). Alternative splicing has also been involved in protein localization as noted for fibronectin (Hynes, 1985), RNA stability, and translational efficiency. Alternative splicing itself is often regulated in a tissue-specific, developmental or temporal manner (Leff *et al.*, 1986). There are many possible combinations to produce an alternatively spliced mRNA. A few examples which have been found in nature are those of splice/don't splice, alternative 5' or 3' splice sites, exon and multiple exon skipping, alternative promoters with alternative 5' splice sites,

and alternative poly A sites with alternative 3' splice sites.

Each of the above alternative splicing patterns are obtained by the selection of either one 5' or 3' splice site over another. This choice involves the influence of many cis and trans-factors. If we consider the donor sites with their relatively well conserved sequences there is only about 5% of them that have an exact match to the consensus sequences which have the strongest use, the other donor sites are usually weaker. This in itself will influence the use of one splice site over another since the closer to the consensus sequence the better the site will be recognized as a splice site. This has been noted for both the 5' and 3' splice sites where mutations convert a weak or strong site into the opposite. However, these sequences are not sufficient and, as noted above, other factors have been found to aid the selection of splice sites during alternative splicing.

In many systems there are non-snRNP trans acting factors that influence splice site selection of the pre-mRNAs. The best characterized regulatory factors are those involved in Drosophila sex determination. The sex-lethal gene product, expressed in females and not male embryos, regulates its own expression as well as that of the Tra gene. In the presence of SXL protein, the TRA protein is produced. Female embryos also produce the TRA-2 protein which together with TRA influence the female-specific splicing of the double-sex gene resulting in female development.

The Drosophila P-element is also regulated at the splicing level. This pre-mRNA has an intron which is removed only in germ cells due to the absence of the soma-specific protein PSI. In somatic cells, the PSI protein interacts with an upstream 5' splice site which promotes the formation of an RNA-protein complex that blocks the binding of U1 snRNP to the accurate 5' splice site and stabilizes U1 snRNP binding to an inactive

pseudo 5' splice site (Siebel *et al.*, 1992). The incompletely spliced pre-mRNA may then be exported to the cytoplasm and translated into a repressor of transposition.

The SR proteins are also involved in splice site selection in some alternative splicing pathways. The SV40 large T and small t products are obtained from the use of two 5' splice sites on the same pre-mRNA. It was noted that in the cell line 293 and its cell extracts that the small t 5' splice site was selected over that of the large T. In contrast, the large T 5' splice site is selected in HeLa cells and extracts (Fu and Manley, 1987; Noble *et al.*, 1987). Further studies involving the addition of SF2/ASF to HeLa S100 nuclear extracts resulted in the preferential use of the proximal 5' splice site over that of the distal large T 5 splice site (Ge and Manley, 1990; Krainer *et al.*, 1990; Mayeda and Krainer, 1992). SC35 has also been shown to be able to influence 5' splice site selection in a similar manner to SF2/ASF.

SR proteins have also been shown to be able to bind not only to the 5' splice site but also to other sequences such as the purine-rich regions found in some exon enhancers. Some examples are that of SF2/ASF binding to the purine-rich splicing enhancer element found in the fibronectin purine-rich sequence of exon EDIIIA (Lavigueur *et al.*, 1993) and in the terminal exon of the bovine growth hormone pre-mRNA (Sun *et al.*, 1993a, 1993b). *In vitro* high-affinity binding site selection studies confirmed that SF2/ASF and SC35 bind to purine-rich sequences (Tacke and Manley, 1995).

As mentioned above the SR proteins are able to stimulate proximal 5' splice site use; however, there is a protein which can antagonize this selection. The hnRNP A1 protein is a non-essential splicing factor that can switch 5' splice site selection from a proximal site to a distal one, an effect opposite to that of SR proteins (Mayeda and

Krainer, 1992; Mayeda *et al.*, 1993; Yang *et al.*, 1994; Cáceres *et al.*, 1994). HnRNP proteins are a group of RNA-binding proteins that contains RRM domains but lack RS domains. The antagonism between the hnRNP and the SR proteins in 5' splice site selection allows for a possible regulatory system based on the competition between the binding of two factors to the same site.

Factors that are involved in splice site selection are beginning to be identified but much work must still be done to distinguish their functions and place all the portions of the puzzle together to arrive with a better picture of how splicing is achieved.

Fibronectin and Alternative Splicing

Fibronectin is a high molecular weight glycoprotein present in the extracellular matrix, plasma, and other body fluids. This glycoprotein has an affinity to collagen, heparin, cell surfaces, bacteria, DNA, and itself. By doing so fibronectin plays a role in attaching cells onto substrata, cell migration, maintenance of normal cell morphology, cell differentiation, and wound healing.

Fibronectin is found as a dimer of two similar polypeptides and exists in at least two forms. Cellular fibronectin is present as an insoluble multimer in the extracellular matrix, while plasma fibronectin is secreted into the plasma by hepatocytes. These different forms vary in their solubility, electrophoretic behavior, some biological functions, and immunogenicity. The polypeptides consist of repeating homology units of 40, 60, and 90 amino acids termed types I, II, and III, respectively.

Many different fibronectin polypeptide variants have been uncovered. These differences are due to the alternative splicing of three regions of the pre-mRNA (Fig. 2). These regions from 3' to 5' are IIICS, EDIIIA (EDI), and EDIIIB (EDII). The IIICS region varies in the number of splicing products from species to species (in rat there are three

products (Schwarzbauer *et al.*, 1983), and in human there are five (Gutman and Kornblihtt, 1987). However, the EDIIIA and EDIIIB regions have only two possible splicing products, the inclusion or exclusion of the alternative exon. In humans, this allows for a possibility of twenty different products from the original fibronectin pre-mRNA. Most of these possible products have been found *in vivo*. These different alternatively spliced products have varying expressions from one tissue to another and in different stages of development. mRNAs encoding cellular fibronectin contain the EDIIIA exon but those encoding plasma fibronectin do not.

Early work done on the alternative splicing of the human fibronectin EDIIIA exon revealed that a 81 nt sequence located inside this exon was necessary for the inclusion of the alternative exon in the mRNA of HeLa cells (Mardon *et al.*, 1987). This region can thus be considered as a splicing enhancer (SE) element. In vitro work done in our laboratory on this 81 nt sequence showed that it stimulates 3' splice site use. The sequence continued to have an effect on the use of the 3' splice site even when it was located closer to the 3' splice site, but lost its effect when located further than 293 nucleotides from the EDIIIA acceptor site. Studies to determine if this element could stimulate splicing on heterologous 3' splice sites revealed an increase in splicing efficiency in the human β-globin substrate and other pre-mRNAs. There was however no stimulation when the 81 nt sequence was inserted into a splicing substrate that was already spliced highly efficiently *in vitro* (Lavigueur *et al.*, 1993).

Deletion studies of this stimulating region revealed the need for a nine nucleotide sequence (GAAGAAGAC) to stimulate the use of the 3' splice site of the EDIIIA exon (Lavigueur *et al.*, 1993). Comparison of fibronectin from different species revealed that there was an equivalent purine-rich sequence in the EDIIIA exon of other species. Other exons, the mouse IgM exon M2 and the cardiac troponin T exon 5, have also been shown to contain purine-rich sequences that stimulated 3' splice site utilization (Watakabe *et al.*, 1993; Xu *et al.*, 1993). Studies that also looked at the exon just downstream of the

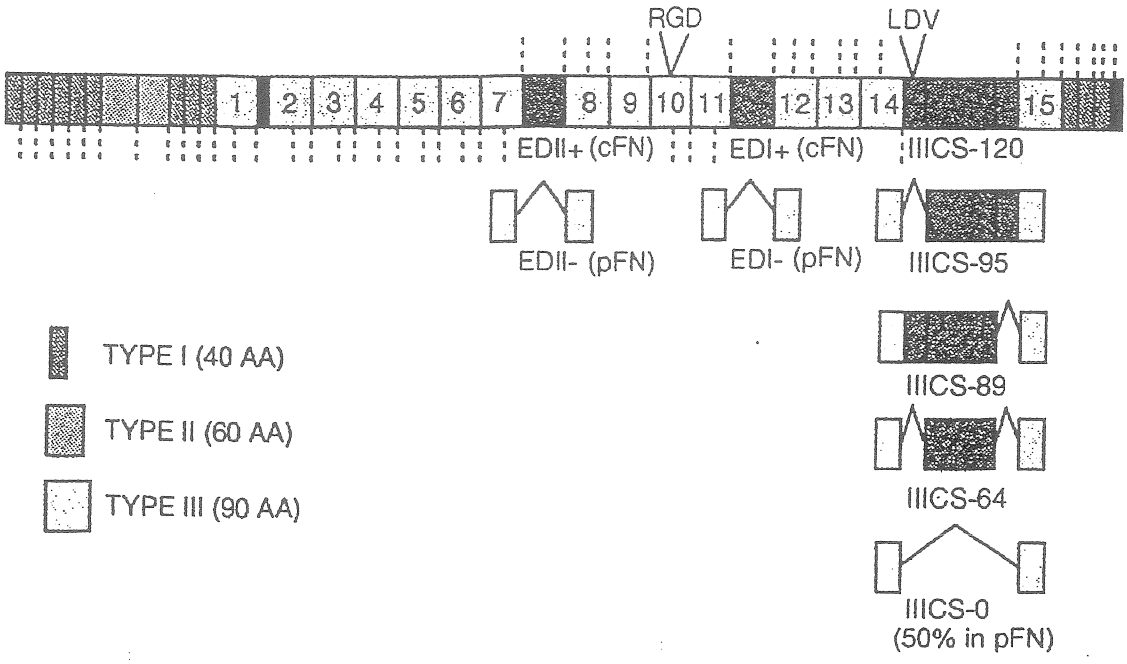


Figure 2: Schematic representation of fibronectin and its variants. The top represents the longest possible fibronectin polypeptide showing internal homologies of types I, II, and III. Constitutive (RGD) and alternatively spliced (LDV) cell-binding sites are indicated. Vertical dashed lines indicate approximate intron positions determined in the rat gene. Type III repeats are numbered from 1 to 15. Numbering excludes EDI and EDII. (Kornblihtt *et al.*, 1996). EDI and EDII are also refered to as EDIIIA and EDIIIB, respectively.

EDIIIA exon (Lavigueur *et al.*, 1993) revealed that there was a similar 9 nt purine-rich sequence in each of the species examined.

To determine if there was an interaction between the SE and any trans-acting factors which could mediate the enhancement of 3' splice site use, competition studies were done with an RNA containing the SE element. These studies revealed that a nuclear factor could bind to the SE element. RNA mobility tests were done to identify binding nuclear factors and revealed that SR proteins interacted with the SE element (Lavigueur *et al.*, 1993). This interaction was not seen when the purine-rich sequence was deleted.

More recent work on the 81 nt SE element has revealed that a second unit may be involved in the negative modulation of exon recognition. This is a short sequence of 5 nucleotides (CAAGG), located 13 nt downstream of the purine-rich sequence (Caputi *et al.*, 1994). Their deletion experiments confirmed the importance of the purine-rich sequence in stimulating 3' splice site utilization. Deletion of the 5 nt sequence (CAAGG) alone led to the production of mRNAs that always contained the EDIIIA exon (Caputi *et al.*, 1994).

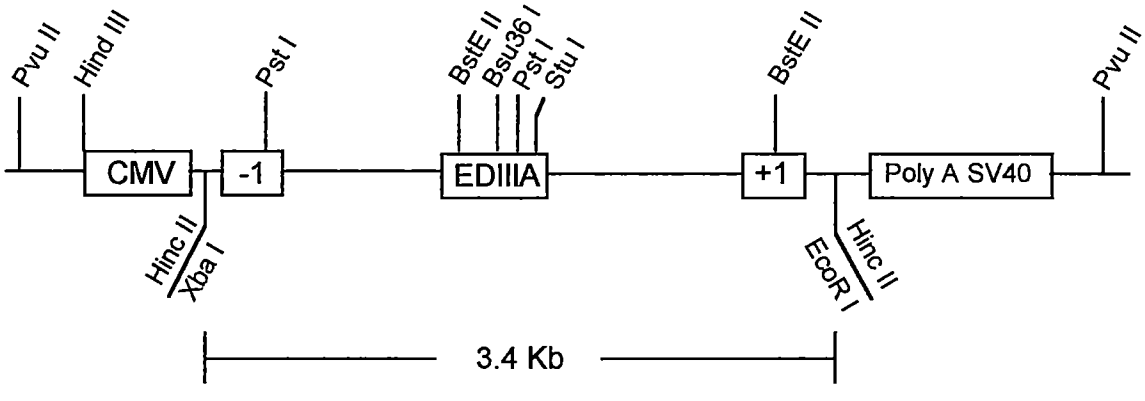
To better understand the alternative splicing mechanism of fibronectin we have chosen the EDIIIA exon and its flanking exons as a system to determine if the purine-rich sequence plays a role in the splicing regulation of EDIIIA *in vivo*. To study this problem we constructed two mini-genes that contain an upstream 5' splice site and the downstream +1 exon of fibronectin along with the EDIIIA exon. In one of the mini-genes the purine-rich region was removed by deleting a 66 base pair region of the exon. These clones were then transfected into two human cell lines which have previously been shown to have different splicing patterns for the endogenous fibronectin. An analysis of the RNA via RT-PCR and sequencing revealed that the original mini-gene did not contain the -1 exon of the fibronectin gene. This mini-gene however showed a similar splicing pattern to that of the endogenous fibronectin and was used to study the role of the purine-rich sequence in the splicing of the EDIIIA exon.

Material and Methods

Mini-gene construction

pCMVFN was generated by inserting a Klenow-filled EcoRI/XbaI fragment (3.4 Kb) from pSP6EDI (provided by Dr Alberto Kornblihtt) into the HincII site of pCMVSV. Based on the restriction map provided, the inserted fragment contained part of exon FNIII-11 (exon -1), exon EDIIIA, and part of exon FNIII-12 (exon +1) of the human fibronectin gene. The pCMVSV vector was made by M. Blanchette and contained the promoter/enhancer region (-525 to +52) from the immediate early gene of the human cytomegalovirus (CMV) and another 400 bp containing the SV40 polyadenylation region.

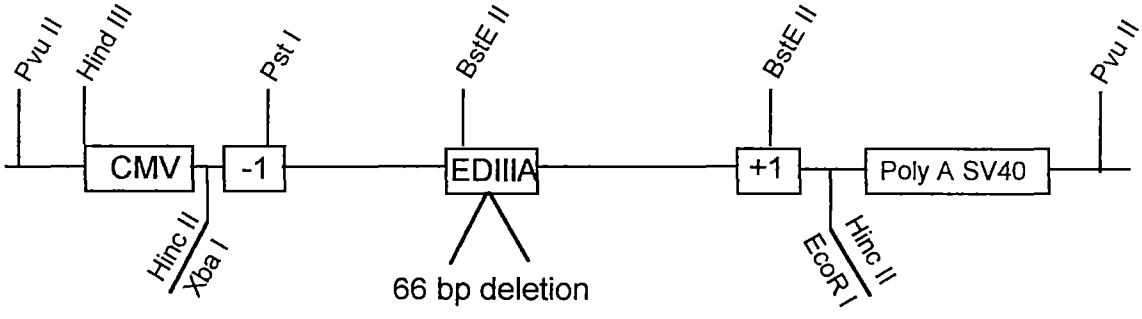
pCMVFN



The pCMVFNΔ vector was made by deleting a 66 bp region, flanked by Bsu36I and StuI, within the EDIIIA exon. After digestion the ends were treated with the Klenow enzyme and ligated together. Verification that the region was deleted was done by

enzyme mapping of this region which looses one of only two PstI site if the deletion was successful.

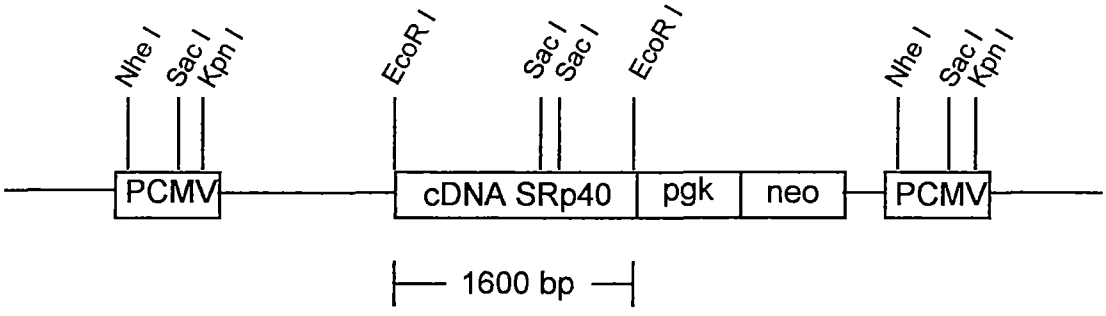
pCMVFN Δ



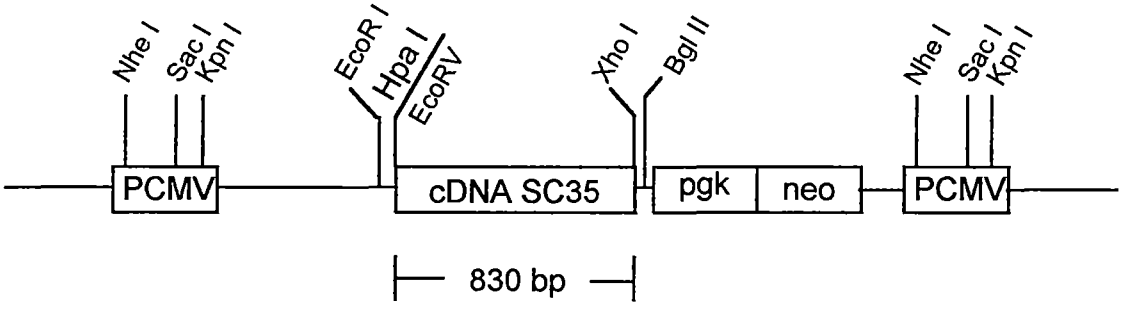
SR cDNA Constructions

To overexpress the SR proteins (SF2, SC35, and SRp40), three vectors were made from the pMSCV 2.1 and 2.2 vectors provided by Dr Robert G. Hawley (Toronto). These vectors contained variant LTRs from the retrovirus CMV, an extended packaging region for high viral titer, a neo gene under the control of an internal phosphoglycerate kinase (pgk) promoter, and a set of unique restriction sites upstream of the pgk promoter. For further information on the pMSCV vectors refer to Hawley *et al.* (1992) and references within. The cDNA of each of the SR proteins were cloned into the unique restriction sites.

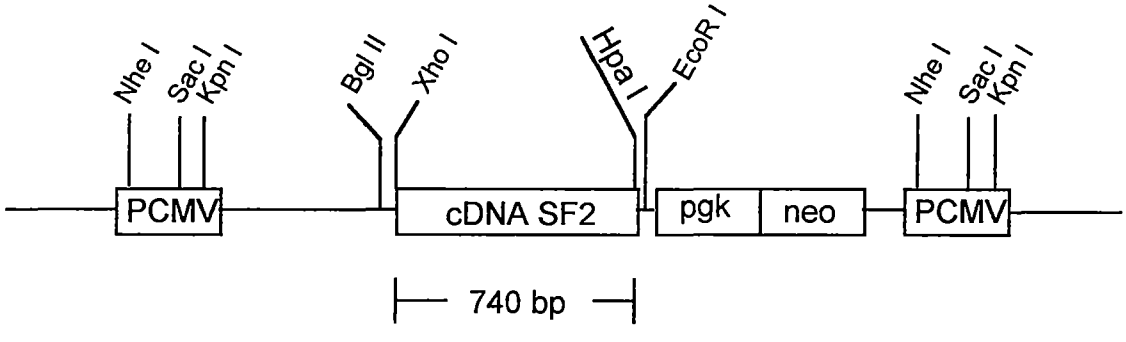
pMSCVSRp40



pMSCVSC35



pMSCVSF2



Plasmid DNA Preparations

Small-scale plasmid DNA preparations were performed using the alkaline lysis technique as described in Sambrook *et al.* (1989). Large-scale plasmid DNA preparations were done by the same alkaline lysis technique but further purification of the DNA was done by cesium chloride density gradient centrifugation (Chabot, 1994).

Restriction Enzyme Analyses

Plasmid DNAs were digested with restriction enzymes purchased from Pharmacia Biotech and New England Biolabs. The reactions were done as specified by the manufacturer.

Isolation and Purification of Fragment and Vector DNAs

The DNA fragments and vector were electroeluted from agarose gels, concentrated and eluted on DEAE columns, extracted with PCA (phenol: chloroform: isoamyl alcohol, 25:24:1), and then precipitated with 2 volumes of 100% ethanol. The samples were resuspended in TE (Tris 10 mM, pH 7.8, EDTA 1 mM). The amount of DNA isolated was analyzed on an agarose gel stained in ethidium bromide.

Klenow Treatment

Some of the inserts and vectors were treated with Klenow before ligation to make the ends compatible. In a 10 µl reaction there was 0.2 - 0.5 µg of DNA along with 1 µl of 10 mM dNTP and 6 units of Klenow enzyme (Pharmacia) and 1 µl of "One for All" 10X buffer (Pharmacia) with the final volume made up with water. Reaction took place at room temperature for 45 min to 1 hr. The Klenow enzyme was then inactivated by heating at 65°C for 10 to 15 min.

Ligation Reactions

Cloning of the DNA into the vectors was performed using the standard method by Sambrook *et al.* (1989). The mixture contained the linearized DNA (0.1 to 0.5 µg), 1.25 mM rATP, 1 µl of T4 ligase (6 U) (Pharmacia), 1 µl of "One for All" 10X buffer, and the volume was made up to 10 µl with water. The molar proportion of vector to insert were between 1:3 and 1:4. The ligation reactions were done at room temperature for 4 to 16 hr (overnight).

cDNAs Sequencing

To identify the 5' splice site of the unknown upstream exon of the mini-gene constructs the spliced products were cloned into the pBluescript vector (Stratagene) and

sequences were obtained by using the T7-sequencing$_{TM}$ kit from Pharmacia. The protocol provided by the manufacturer was used.

Transformation

5 µl of the ligation mix was added to 100 µl of competent cells (*E.coli* DH5α) and placed on ice for 30 min. The mixture was then incubated at 42°C for 90 s and then placed back on ice for 2 min. To this mix, 500 µl of LB growth culture (10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 10 g NaCl in 1 letter of water) was added and the samples were placed at 37°C for 30 min. 200 µl of this mix was spread onto agar petri dishes containing 100 µl/ml of ampicillin. The plates were placed in a 37°C incubator for the night. The next day colonies were picked and incubated in 2 ml of LB containing ampicillin at 37°C with agitation.

Transfections

Transfections were done using the calcium phosphate precipitation method as done by Graham and Van der Eb (1973). Both WI38VA13 and MRC5 cells were grown to 50% confluency for the transfection. WI38VA13 cells derived from WI38 human lung cells transformed by SV40 viruses and the MRC5 cells are derived from normal lung tissue and have a fibroblast-like morphology. To 500 µl of CC+ (100 ml TE: 1 mM Tris pH 7.5 and 0.1 mM EDTA, 15 ml 2M $CaCl_2$, and 375 µl of 10 mg/ml sonicated calf thymus DNA or salmon sperm DNA), was added 10 µg of the DNA to transfect in 50 µl

of TE (10:1) and kept on ice. At room temperature 550 µl of 2X HEBS solution (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM Hepes pH 7.12) was added to the mixture which was incubated for 5 to 10 min. This solution was then applied dropwise to 5-10 ml of medium in the petri dishes that contained the cells and was allowed to sit for 4 hr in the incubator at 37°C.

After this incubation period the medium was removed and 4 ml of DMEM + 20% glycerol was added to the cells and incubated at 37°C for 2 min. This solution was then removed and the cells were rinsed twice with 10 ml of DMEM. 10 ml of DMEM + 10% FBS was added and the cells were incubated at 37°C for 48 hr before extracting the RNA.

Stable Transfection Assays

The same protocol was used as for the transient transfections. Following the 48 hr incubation the cells were split to 1/4 the original amount and the selection was done with G418 (400 µg/ml, GIBCO) for one to two weeks before isolating the RNA and proteins.

Total RNA Extraction

RNA was extracted from the different cell lines using the guanidinium thiocyanate extraction protocol (Chabot, 1994). Any contaminating plasmid DNA was removed by treating the RNA samples with 3.75 units of DNase I (Pharmacia) in the presence of 20 units of RNAguard (Pharmacia) and 4 mM DTT for 15 min at 37°C. The samples were

extracted with PCA and the RNA precipitated in ethanol. The final concentration of the RNA was determined on agarose gels stained with ethidium bromide.

RT-PCR

2.0 to 2.5 μg of RNA was reverse transcribed for 1 hr at 37°C into cDNA with 3 units of AMV-reverse transcriptase (Promega) in a buffer (50 μl) containing 10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 50 mM KCl, 260 μM of each dNTP, 30 μg/ml of BSA, 4 μM DTT, 206 units/ml of RNAguard and 2 μg/ml of the appropriate downstream oligonucleotide. The downstream oligonucleotide was incubated with the RNA in the buffer at 50°C for 3 min and then at 30°C for 1 min prior to the addition of the AMV-reverse transcriptase. A mixture containing 50 ng of each oligonucleotide, 3 μCi of radiolabeled [α-^{32}P]dCTP (800 Ci/mmole; Amersham) and 1.25 units of Taq DNA polymerase (Pharmacia) was then added. The 'touchdown' protocol was used to amplify the cDNA (protocol noted below). The products were separated by electrophoresis on 5% polyacrylamide gels and the gels were dried and exposed on film.

The sequences of the oligonucleotides used in the RT-PCR assays were:

FN III-11: 5' AGGCTTGCAGCCCACAGTGGA 3'

FN III-12: 5' GGGGTCACCCGCACTCGATAT 3'

FN1495: 5' GAGGGGCTGGCTCTCCATAT 3'

FN1395: 5' ATAAGGGACGTGGAC 3'

CMV-1: 5' AGACGCCATCCACGCTGTTT 3'

PCR Touchdown Program (Modified from Don *et al.*, 1991)

1.	94°C, 1,5 min.	70°C, 2 min.	72°C, 3 min.
2.	94°C, 1,5 min.	69°C, 2 min.	72°C, 3 min.
3.	94°C, 1,5 min.	67°C, 2 min.	72°C, 3 min.
4.	94°C, 1,5 min.	65°C, 2 min.	72°C, 3 min.
5.	94°C, 1,5 min.	63°C, 2 min.	72°C, 3 min.
6.	94°C, 1,5 min.	61°C, 2 min.	72°C, 3 min.
7.	94°C, 1,5 min.	59°C, 2 min.	72°C, 3 min.
8.	94°C, 1,5 min.	57°C, 2 min.	72°C, 3 min.
9.	94°C, 1,5 min.	55°C, 2 min.	72°C, 3 min.
10.	94°C, 1,5 min.	53°C, 2 min.	72°C, 3 min.
11.	94°C, 1,5 min.	51°C, 2 min.	72°C, 3 min.
12.	94°C, 1,5 min.	50°C, 2 min.	72°C, 3 min.
13.	Go to step 12 and repeat 30X (This step was also done at 25X in some cases).		
14.	72°C, 15 min.		
15.	4°C, 15 hr.		
16.	End.		

Protein Extraction

The cells were collected and centrifuged from confluent petri dishes, rinsed twice with a cold PBS solution and then resuspended in 2X SDS gel loading buffer (100 mM

Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol). The samples were boiled for 5 to 10 min then stored at -80°C.

Western Blot Assays

The protein samples were boiled for 5 min prior to loading onto the gel. The proteins were separated on a 12.5% polyacrylamide/SDS gel and transferred to a Hybond-C nitrocellulose membrane. Once the transfer had been accomplished the membrane was rinsed in a TBS solution (20 mM Tris-HCl, 137 mM NaCl), for 5 min. Then the membrane was placed in a solution of 5% powdered milk in TBST (TBS + 0.1% Tween 20) and incubated at room temperature for 1 hr. The membrane was washed twice for 15 min each in TBST (Note that all solutions used contained 20 mM β-glycerophosphate). The primary detection was done with a solution of hybridoma producing the mAb 104 antibody specific for the phosphorylated SR protein. Incubation in the presence of the antibody was done for 1 hr. Three washes were done in the TBST solution for 10 min each. The second detection was done with a solution of 5% powdered milk/TBST containing the anti-mouse Ig, horseradish peroxidase linked whole antibody at a dilution of 1 in 1000. Incubation was done for 1 hr at room temperature. The membrane was rinsed 4 times with the TBST solution before performing the detection. The detection was done using the ECL western blotting protocol (Amersham) and the membrane was exposed to an XRP Kodak film.

Results

Pre-mRNAs expressed from a transfected mini-gene follows a splicing pattern similar to that of the endogenous fibronectin pre-mRNA

Previous studies have revealed that the fibronectin pre-mRNA undergoes alternative splicing in three regions allowing for the production of several different isoforms of the fibronectin protein (Schwarzbauer *et al.*, 1983; Kornblihtt *et al.*, 1984). Alternative splicing of fibronectin pre-mRNA varies between different cell lines. An example of this involves the two cell lines MRC5 and WI38VA13. The frequency of inclusion of the alternative EDIIIA exon in WI38VA13 cells is greater than in MRC5 cells. To verify that pre-mRNAs derived from mini-gene construct pCMVFN are spliced in a manner similar to endogenous fibronectin pre-mRNAs, RT-PCRs were done on RNA extracts of transfected WI38VA13 and MRC5 cells. In Figure 3, the frequency of inclusion and exclusion of exon EDIIIA, referred to as inclusion or exclusion product in the text, of the endogenous fibronectin is shown. There is relatively more inclusion product in WI38VA13 cells (Fig. 3, lane 1) than in MRC5 cells (lane 5). We compared these results with those obtained following transient fibronectin expression from the mini-gene construct pCMVFN (Fig. 3, lanes 3 and 7). The profile obtained suggests that there is also relatively more inclusion of the EDIIIA exon from the exogenous pre-mRNA in WI38VA13 cells (Fig. 3, lane 3) than in MRC5 cells (lane 7). The relative level of exclusion products from the endogenous and exogenous fibronectin pre-mRNA appears to be approximately equivalent.

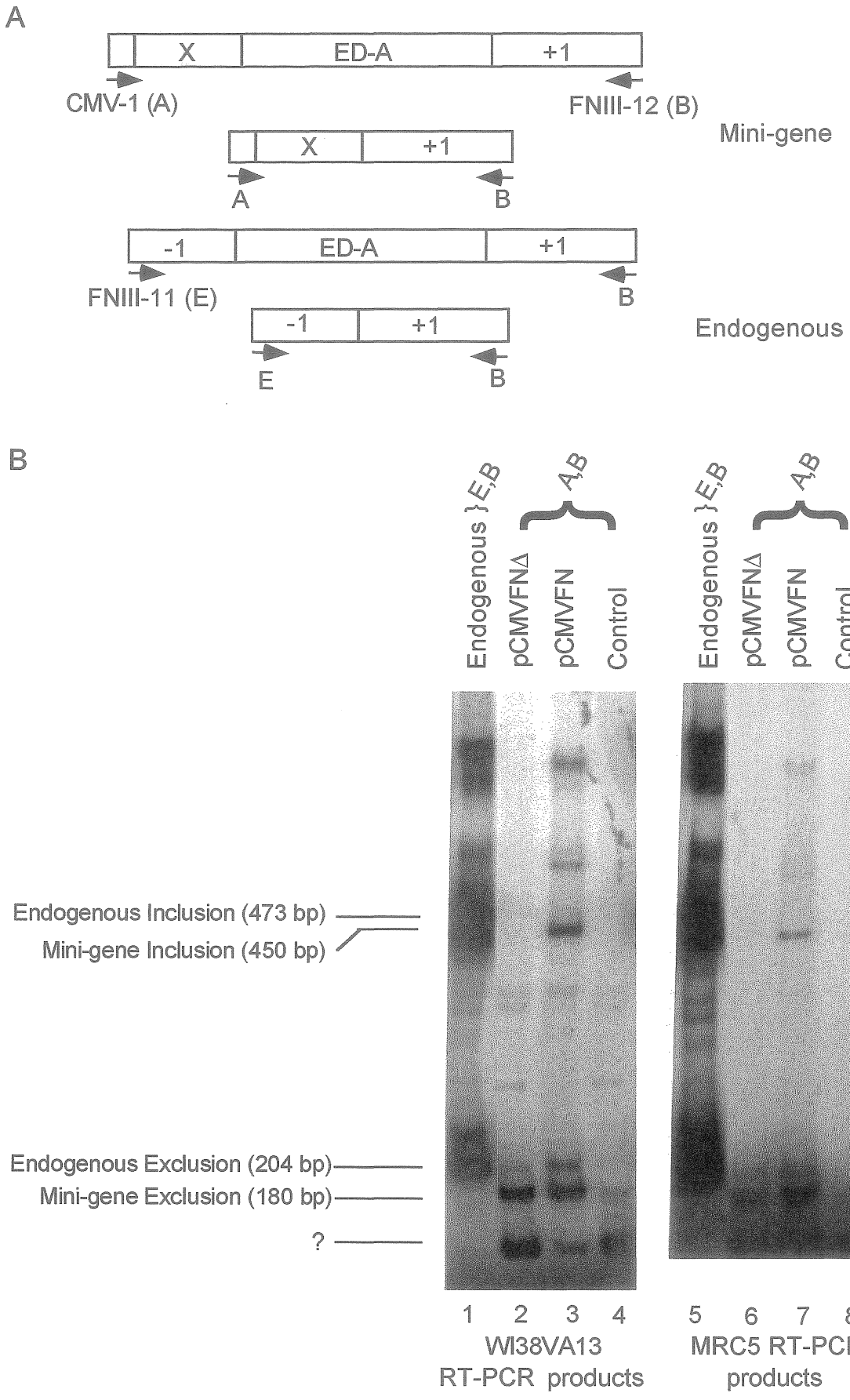
There was however a problem concerning the size of the amplified products derived from the transfected mini-gene. These were shorter than expected and should have migrated slower than the amplified materials derived from the endogenous products. We expected an inclusion product of 616 bp and an exclusion product of 347 bp. However, the products obtained were at approximately 450 bp and 180 bp. The difference between these two products suggest that they differ from the inclusion of the EDIIIA exon of 270 bp. The fact that the products do not have the expected sizes may indicate a problem with their migration in the gel or may be due to the use of an aberrant splice site. The region immediately downstream of the CMV promoter in pCMVFN and thought to correspond to exon -1 and flanking intron sequences had not been sequenced in our laboratory. However, the size of the inserted fibronectin fragment fitted the expected size for a fragment containing the -1 to +1 region. To determine if the amplified products corresponded to the inclusion and exclusion products, a set of digestions was carried out on the RT-PCR products. Secondly, to determine if proper splice site utilization had occurred, we subcloned the RT-PCR products into the pBluescipt KS+ vector and sequenced them.

Digestions of the RT-PCR products

Exon EDIIIA contains some unique restriction enzyme sites that were used to identify the inclusion products amplified by RT-PCR. In Figure 4, the RT-PCR sample derived from WI38VA13 transfected cells was digested with either Bsu36 I (lane 2) or Stu I (lane 3), which cut only in the EDIIIA exon. Another digestion was done with Pst I

Figure 3: RT-PCR products derived from splicing of the endogenous and transfected fibronectin genes.

Both WI38VA13 and MRC5 cells were transfected with the pCMVFN and pCMVFNΔ constructs. Total RNA was extracted and amplified by RT-PCR. These extracts were compared to RNA from untransfected cells to verify that the mini-gene pCMVFN had a similar splicing pattern to that of the endogenous fibronectin. A) Different primers specific for the transfected and endogenous fibronectin were used in the PCR reactions. B) Lanes 1 and 5 display the amplification products for the endogenous fibronectin (inclusion product at 473 bp and exclusion product at 204 bp). Note that the relative amount of inclusion product is greater in WI38VA13 cells than in MRC5 cells. Amplification products derived from the transfected mini-gene pCMVFN indicate that more inclusion product is produced in WI38VA13 than in MRC5 cells (lanes 3 and 7, inclusion product at 450 bp and exclusion product at 180 bp). Lanes 2 and 6 contain the amplification products derived from the transfected mini-gene construct pCMVFNΔ (deletion of the purine-rich sequence). The predicted size of the inclusion product should be 384 bp. In lanes 2 and 6, only the exclusion product is detected (band at 180 bp). Negative controls were done by using the primers that were specific for the transfected mini-genes to amplify the RNA from untransfected cells RNA (lanes 4 and 8). The band below 180 bp (?) is thought to represent an artefact of PCR since it appears also in untransfected extracts. The position and size of the amplified products are shown.


A
X
ED-A
+1
CMV-1 (A)
FNIII-12 (B)
Mini-gene
X
+1
A
B
-1
ED-A
+1
FNIII-11 (E)
B
-1
+1
E
B
Endogenous
B
Endogenous
E,B
pCMVFNΔ
A,B
pCMVFN
Control
Endogenous
E,B
pCMVFNΔ
A,B
pCMVFN
Control
Endogenous Inclusion (473 bp)
Mini-gene Inclusion (450 bp)
Endogenous Exclusion (204 bp)
Mini-gene Exclusion (180 bp)
?
1 2 3 4
WI38VA13
RT-PCR products
5 6 7 8
MRC5 RT-PCR
products

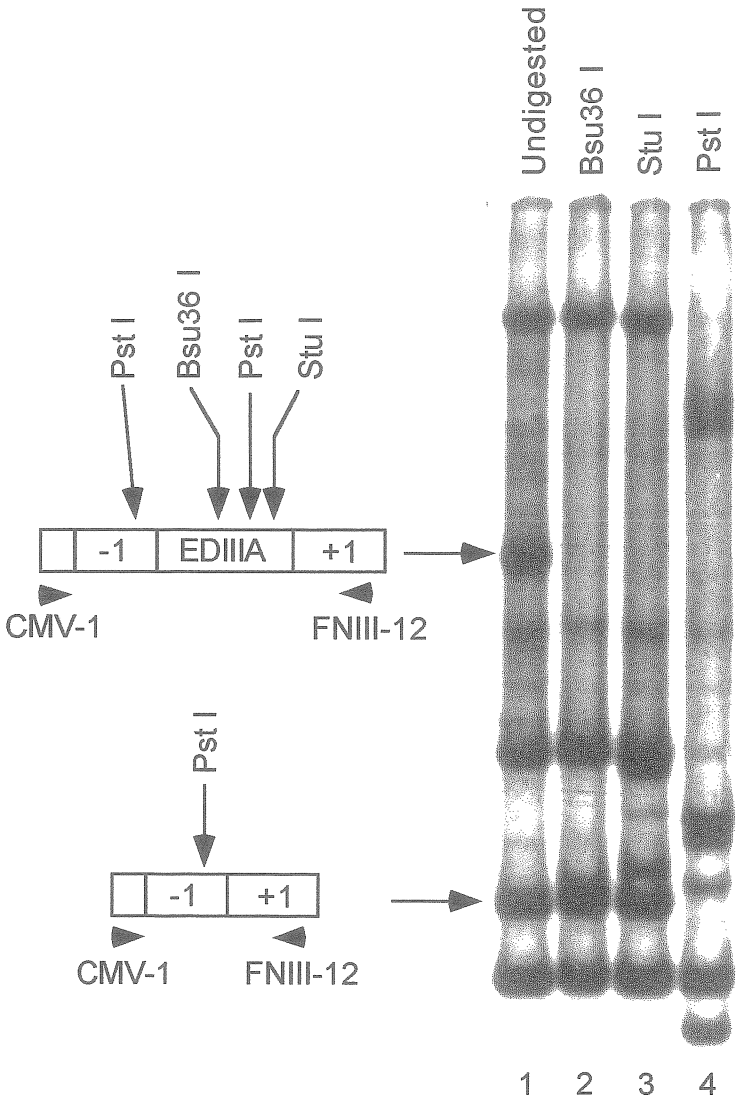
lane 4), which cuts in both the EDIIIA exon and the -1 exon of fibronectin. In the undigested sample (Fig. 4, lane 1), both the inclusion and exclusion products are visible. After digestion with either Bsu36 I and Stu I, the inclusion band was lost. In lanes 1 through 3 the exclusion product was always present. However, both the inclusion and exclusion products were lost in the digestion by Pst I (Fig. 4, lane 4). This suggests that the bands thought to represent inclusion and exclusion products were indeed so even though their sizes were not as expected. The fact that the band immediately below the putative exclusion product was not affected by any digestion suggests that this band represents an artifactual product of RT-PCR amplification.

Sequencing the RT-PCR products

To characterize these amplified products more precisely, their sequence was obtained. The RT-PCR products were run on a 2% agarose gel containing ethidium bromide so as to be able to isolate the inclusion and exclusion products. In Figure 5B, lanes 2 and 3 contain the exclusion RT-PCR products from the WI38VA13 and MRC5 cell, respectively. The inclusion products were however not detected on this agarose gel using the original set of primers CMV-1 and FNIII-12 shown in Figure 5A. To overcome this problem, we used a second set of primers that were specific for the inclusion products (Fig. 5A, primers A and D). This new set of primers amplified a fragment of 222 bp noted in Figure 5B, lanes 4 and 5. The five indicated bands were cut out, electroeluted from the agarose gel and subcloned into the pBluescript KS+ vector. Note that two artifactual bands that could also correspond to the exclusion products in both cell lines

Figure 4: Digestions of the RT-PCR amplification products

To confirm the identity of the amplified products seen in Figure 3, I performed restriction enzyme digestions following RT-PCR assays. Lane 1 represents the undigested products of a RT-PCR reaction performed on a WI38VA13 sample with the CMV-1 and FNIII-12 primers. The digested samples (lanes 2 to 4) reveal either the loss of the inclusion product (digestion by *Bsu36I* lane 2, or *StuI* lane 3) or the loss of both inclusion and exclusion products (digestion by *PstI* lane 4). A diagram of the expected amplification product and the position of the primers is shown on the left of the gel.


Undigested
Bsu36 I
Stu I
Pst I
Pst I
Bsu36 I
Pst I
Stu I
-1
EDIIIA
+1
CMV-1
FNIII-12
Pst I
-1
+1
CMV-1
FNIII-12
1
2
3
4

were also isolated and subcloned (bands 2 and 4). The inclusion product for the MRC5 cells (Fig 5B, lane 5) was not subcloned into pBluescript KS+ because there was too little product obtained from the RT-PCR.

Exclusion Products

Figure 6 represents a portion of the sequences obtained from the subcloned RT-PCR fragments. The cDNA sequences of the spliced endogenous fibronectin was compared to the sequences obtained from the RT-PCR products. The possible exclusion products obtained from the sequencing of bands 1 through 4 revealed two different sets of identical sequences. Bands 1 and 3 yielded the same sequence which includes the complete sequence of exon +1 (Fig. 6B). The sequence upstream of exon +1 is however not that of the expected exon -1. This indicates that either exon -1 is absent from the construct and/or that an aberrant 5' splice site had been used in an unknown region of the mini-gene. This aberrant 5' splice site was spliced to the authentic 3' splice site of exon +1.

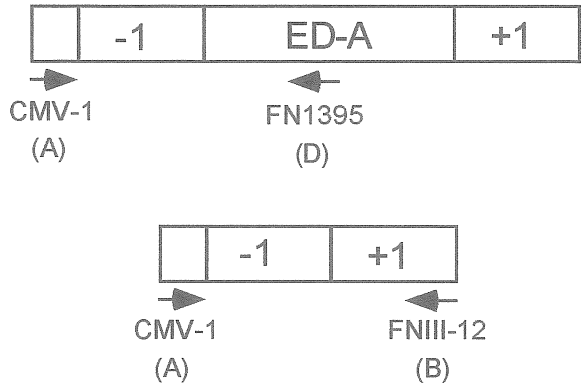
Inclusion Products

The inclusion product from the WI38VA13 cell line (band 5) contains the EDIIIA exon sequence starting at position +1 of the exon (Fig. 6A). The upstream sequence was identical to that of the exclusion products of bands 1 and 3 (compare with clone 1 and 3 in 6B). Thus, the inclusion product seemed to have been produced from the use of the same aberrant 5' splice site but in this case splicing has occurred to the 3' splice site of exon EDIIIA. To identify the origin of these sequences that were found spliced either to exon EDIIIA or +1, we compared these sequences with the sequence surrounding the CMV promoter (supplied by Marco Blanchette, personal communication). This analysis

Figure 5: Isolation of inclusion and exclusion products

A) Diagram indicating the position of the primers used to amplify the products in the RT-PCR assays. B) The RT-PCR products were separated on a 2% agarose gel containing ethidium bromide. Two possible exclusion products are present around 180 bp (indicated 1,2 and 3,4 for the WI38VA13 and MRC5 cells, respectively). Because the inclusion products were not seen in this gel with this combination of primers, a second set of primers was used (primers FN1395 and CMV-1) to produce a band at 222 bp (indicated 5 in lane 4 for the WI38VA13 cells). There is a faint band at 222 bp for the MRC5 cells (lane 5) which we believe to be identical to that of the WI38VA13 cells.

A



Band 5

Bands 1 and 3
or 2 and 4 ?

B

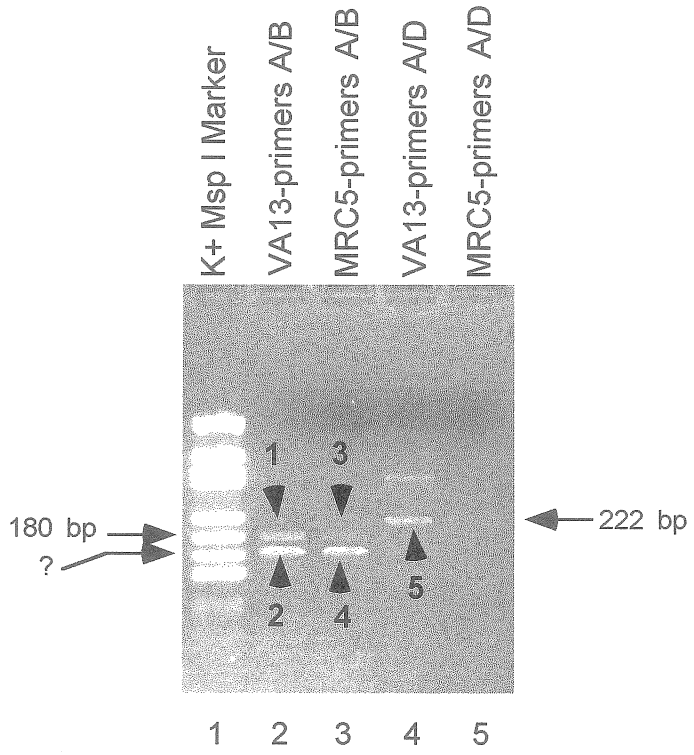


Figure 6: Sequences of the RT-PCR amplified products

The bands indicated 1 to 5 in Figure 5 were cut out of the gel, electroeluted, and cloned into pBluescript KS+. Using the CMV-1 primer, the sequences of these subcloned fragments were obtained. Figure 6A displays part of the sequence obtained for the inclusion product (band 5) compared to the predicted cDNA sequence. Figure 6B shows the two sequences for the exclusion product (bands 1 and 3) compared to the cDNA sequence of the endogenous exclusion product. The two other possible exclusion products were found to be artifacts of the PCR and their sequences were identical (Figure 6C). A search of GeneBank revealed that their sequence was homologous to the 28S ribosomal RNA sequence. Figure 6D shows part of the sequence of the mini-gene construct of pCMVFN to indicate the presence of a putative 5□ splice site 34 nt downstream of the CMV promoter.

A) INCLUSION PRODUCTS

EDIII-A EXON

cDNA clone 5: *ACTGCAGGTCCTAGAGGATCTTAG***ACATTGACTGCCCTAAAGGA**

cDNA fibronectin: CTCTGGTCAGACTGCAGTAACCA**ACATTGATCGCCCTAAAGGA**

-1 EXON

B) EXCLUSION PRODUCTS

+1 EXON

cDNA clone 1: AC*CGACTCTAGACTGCAGGTCCTAGAGGATCTTAG***CTATTCCT**

cDNA clone 3: AC*CGACTCTAGACTGCAGGTCCTAGAGGATCTTAG***CTATTCCT**

cDNA fibronectin: AGAGAGTCAGCCTCTGGTTCAGACTGCAGTAACCA**CTATTCCT**

-1 EXON

C) 28S RIBOSOMAL RNA

cDNA clone 2: GAGTGTCTTGGGGCCGAAACGACTCAACCTATTCTCAAACTTTAAAT

cDNA clone 4: .....AAACGACTCAACCTATTCTCAAACTTTAAAT

D) SEQUENCE DOWNSTREAM OF THE CMV PROMOTOR

AGAAGACAC*CGACTCTTAGACTGCAGGTCCTAGAGGATCTT**AGGTAACA**TGA

CMV PRIMER

5' splice site

revealed that the expected sequence of exon -1 was absent but a sequence of unknown origin was instead present. Examining these sequences revealed that there was a putative 5' splice site sequence 34 nt downstream of the CMV promoter (Fig. 6D). Because the sequence upstream of the 5' splice site are present in clones 1, 3 and 5, this 5' splice site sequence was most likely used to generate inclusion and exclusion products from the mini-gene.

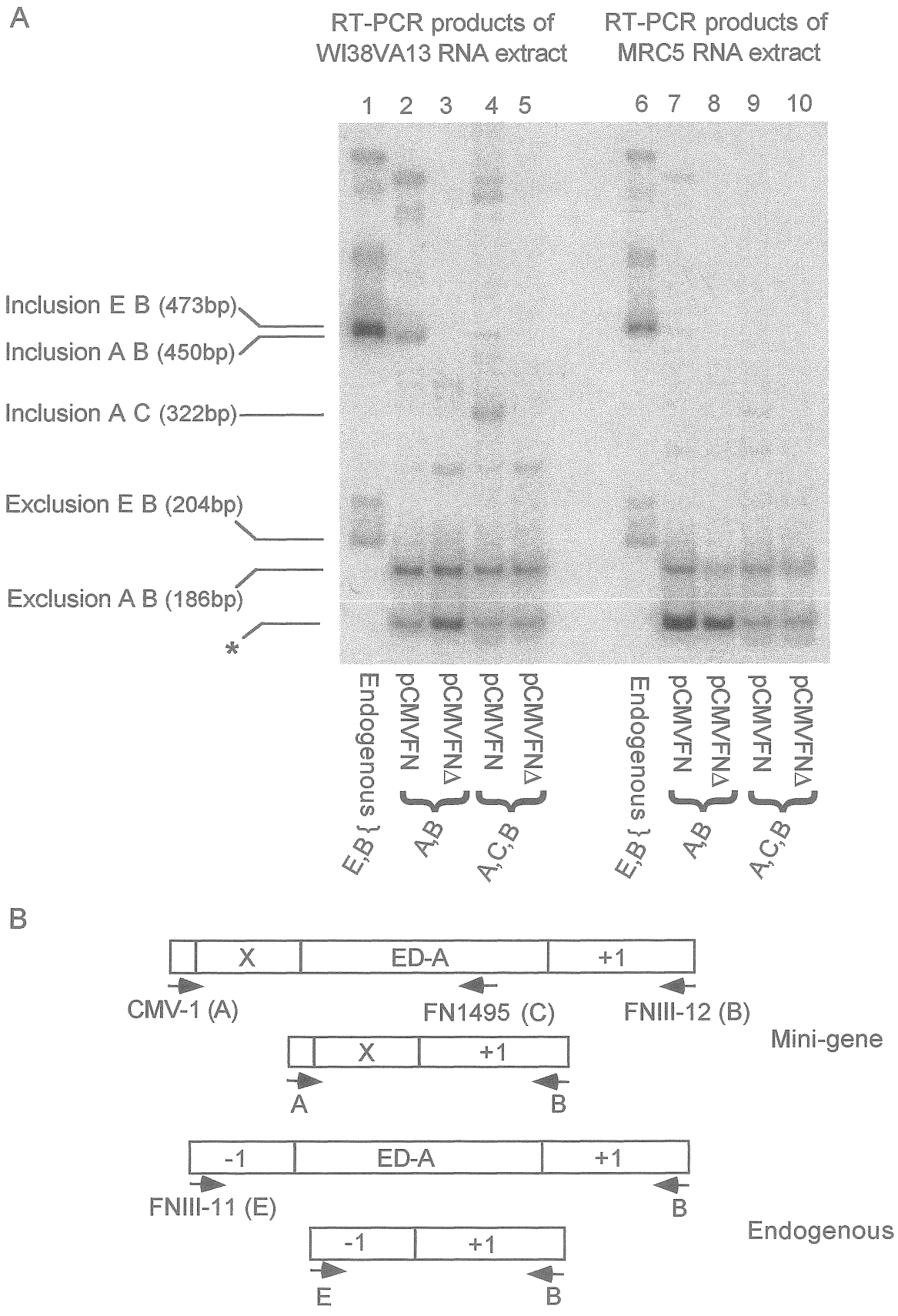
Finally, the sequencing of bands 2 and 4 revealed that the corresponding amplified products were derived from a sequence homologous to the 28S ribosomal RNA (when compared to the sequences in GenBank). Because these bands were present in all of the RT-PCR assays and were devoid of Pst I, Stu I, and Bsu36 I restriction sites (see Fig. 4), our results indicate that these bands are PCR artifacts unrelated to fibronectin splicing.

Deletion of the EDIIIA splicing enhancer leads to exon EDIIIA skipping

Both the fibronectin mini-gene and a fibronectin mini-gene containing a deletion of the splicing enhancer in the EDIIIA exon were transfected into MRC5 and WI38VA13 cells. Two days after transfection, the RNA was isolated and RT-PCR reactions were carried out as before to determine the frequency of exon EDIIIA inclusion. For these RT-PCRs, two sets of primers were used to identify the inclusion products. The first is the original set including primers FNIII-12 and CMV-1 (Fig. 3), whereas the second set included these two primers as well as a third primer, FN1495, represented in Figure 7B. This was done to overcome the problem encountered in the production of the cDNAs which were subcloned for sequencing, such that we would obtain an inclusion product of

Figure 7: RT-PCR assay to determine the change in the level of inclusion product after deletion of the splicing enhancer.

A) Two sets of primers were used to identify the inclusion product. For the endogenous fibronectin, the relative amount of inclusion product in WI38VA13 cells is greater than in MRC5 cell (band at 473 bp lanes 1 and 6). The same result is obtained with the transfected pCMVFN gene (lanes 2, 4, 7, and 9). The exclusion product is at 204 bp. Deletion of the 66 bp region containing the purine-rich sequence (pCMVFNΔ) promotes the disappearance of the inclusion product in both WI38VA13 (lanes 3 and 5) and MRC5 cells (lanes 8 and 10). The expected band sizes for the inclusion products in lanes 3 and 8 should be 384 bp and in lanes 5 and 10, 256 bp. * represents the artifactual amplification product. B) Diagram of the primers used to amplify the spliced products.


A
RT-PCR products of WI38VA13 RNA extract
RT-PCR products of MRC5 RNA extract
1 2 3 4 5
6 7 8 9 10
Inclusion E B (473bp)
Inclusion A B (450bp)
Inclusion A C (322bp)
Exclusion E B (204bp)
Exclusion A B (186bp)
*
Endogenous
pCMVFN
pCMVFNΔ
pCMVFN
pCMVFNΔ
Endogenous
pCMVFN
pCMVFNΔ
pCMVFN
pCMVFNΔ
E,B
A,B
A,C,B
E,B
A,B
A,C,B
B
X
ED-A
+1
CMV-1 (A)
FN1495 (C)
FNIII-12 (B)
Mini-gene
X
+1
A
B
-1
ED-A
+1
FNIII-11 (E)
B
Endogenous
-1
+1
E
B

323 bp as well as or instead of the 457 bp product.

The results are shown in Figure 7A. First, we compared the frequency of splicing for the complete mini-gene in the two cell types MRC5 and WI38VA13 (WI38VA13, lanes 2 and 4; MRC5, lanes 7 and 9). As observed for endogenous fibronectin (lanes 1 and 6), the relative level of inclusion product in WI38VA13 cells was greater than in MRC5 cells. Deletion of a 66 bp region within the EDIIIA exon which contains the splicing enhancer element led to the loss of the inclusion product in both cell lines (Fig. 7A, lanes 3, 5, 8, and 10; see also Fig. 3, lanes 2 and 6). Amplification of the exclusion product was still observed. This result indicates that this region contains a sequence needed for the inclusion of the EDIIIA exon, consistent with previous observations (Mardon *et al.*, 1987).

Expression of SR proteins in MRC5 cells

To determine which SR protein (or proteins) was involved in regulation of EDIIIA exon inclusion, MRC5 cells were transfected with plasmids that contained the cDNAs of one of three SR proteins (SF2, SC35, and SRp40) under the control of a CMV promoter. These plasmids (pMSCVSC35, pMSCVSRp40, and pMSCVSF2) also contain the gene for the resistance to neomycin so that a selection could be done on the transfected cells with a medium containing G418. A stable population of cell was obtained for the transfection of SRp40 but attempts at obtaining stable transfectants with pMSCVSF2 and pMSCVSC35 were unsuccessful.

To verify the expression of the SRp40 protein in MRC5 cells, a protein extract of these cells was compared with that of untransfected cells. Proteins were separated on a 12.5% polyacrylamide/SDS gel, transferred to a nitrocellulose membrane, and probed with the mAb104 antibody which detects phosphorylated SR proteins. Figure 8 represents the profile of the SR proteins in different cell lines. WI38VA13 cells do contain more SC35, SF2, and SRp40 than MRC5 cells (lanes 1 and 2, respectively), confirming a previous report by Chabot *et al.* (1992). Comparing MRC5 cells with the G418-resistant MRC5 cells which should overexpress SRp40 (lanes 2 and 3), we found to our surprise that there was a decrease in the amount of phosphorylated SRp40 protein. All other SR proteins were found in approximately the same amount as that of the normal MRC5 cells. The reason for this reduction in SRp40 remains unknown.

To see if this reduction in phosphorylated SRp40 expression had any effect on the splicing pattern of the fibronectin pre-mRNA, RT-PCR reactions were done for the endogenous fibronectin. Compared to untransfected MRC5 cells, there was less inclusion of the EDIIIA exon in the G418-resistant MRC5 cells that displayed a reduction in the expression of the SRp40 protein (Fig. 9).

This decrease in the inclusion of the EDIIIA exon may be due to the decrease in the amount of phosphorylated SRp40 protein. In other words, if the phosphorylated SRp40 protein plays a role in exon EDIIIA inclusion a decrease in the amount of phosphorylated SRp40 protein would lead to an expected reduction in the inclusion of EDIIIA.

Figure 8: Western Blot assay for the SR proteins

MRC5 cells were transfected with a plasmid which contains the cDNA of the SRp40 protein. The plasmid used (pMSCVSRp40) also contains the neomycin resistance gene so that selection of stable transfectants could be achieved. Protein extracts were separated on a 12.5 % acrylamide/SDS gel, transferred to a nitrocellulose membrane, and detected with mAb104, an antibody specific for phosphorylated SR proteins. WI38VA13 cells (lane 1, non-transfected) express a greater amount of SR proteins SF2, SC35, and SRp40 than MRC5 cells (lane 2, non-transfected). Note that SC35 and ASF/SF2 co-migrate in this gel. After selection with G418, SR proteins from the MRC5 cells transfected with the SRp40 cDNA were analyzed (lane 3). We observe a decrease in the amount of phosphorylated SRp40 protein in MRC5 cells that were expected to overexpress SRp40.

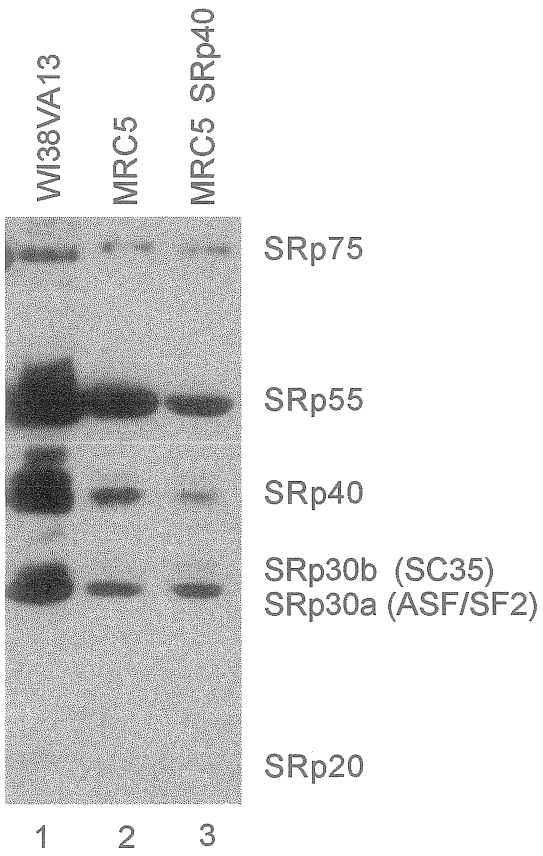
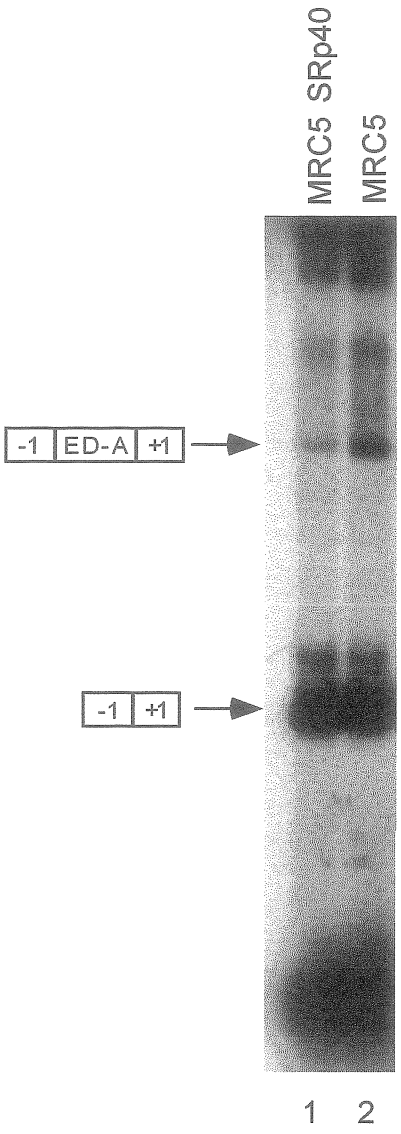

WI38VA13
MRC5
MRC5 SRp40
SRp75
SRp55
SRp40
SRp30b (SC35)
SRp30a (ASF/SF2)
SRp20
1
2
3

Figure 9: RT-PCR amplification products from the endogenous fibronectin mRNAs in MRC5 cells stably transfected with the cDNA of SRp40

A decrease in the amount of inclusion of the EDIIIA exon is noted in the MRC5 cells that were transfected with the SRp40 cDNA when compared to non-transfected MRC5 cells (lanes 1 and 2, respectively). Schematic representations of the products are shown to the left of the gel. The primers used to amplify the products were FNIII-11 and FNIII-12.


MRC5
SRp40
MRC5
-1
ED-A
+1
-1
+1
1
2

Discussion

The objective of this work was to determine whether the purine-rich splicing enhancer played a role in the regulation of the alternative splicing of the fibronectin EDIIIA exon in MRC5 and WI38VA13 cells. WI38VA13 cells spliced the endogenous fibronectin pre-mRNA to yield more inclusion product than MRC5 cells. Using a fibronectin mini-gene construct, we first demonstrated that inclusion of the EDIIIA exon followed a pattern that was similar to that of the endogenous fibronectin in both cell lines. However, the abnormal migration of the RT-PCR products suggested that an aberrant splice site had been used in the mini-gene. Further characterization of the RT-PCR products indicated that the exon upstream of EDIIIA was absent from the mini-gene, being replaced by an unknown sequence carrying a 5' splice site. Fortunately however, the pre-mRNA expressed from this hybrid mini-gene was spliced to yield a ratio of inclusion/exclusion similar to the endogenous fibronectin pre-mRNA. This result indicates that exon -1 and its associated 5' splice site are not required to obtain regulated splicing.

Using this mini-gene and a derivative carrying a deletion of the splicing enhancer, we next addressed the effects of the deletion following transfection in WI38VA13 and MRC5 cells. The rationale behind this experiment was to determine whether the splicing enhancer represented a general stimulator of splicing or if it was involved in the regulation of EDIIIA splicing. In the case of a general splicing stimulator, the deletion of the splicing enhancer is predicted to lead to an equivalent decrease in inclusion in both WI38VA13 and MRC5 cells. In contrast, if the splicing enhancer is involved in

regulation, the decrease in inclusion is predicted to be more important in WI38VA13 cells, possibly becoming equivalent to the level of inclusion in MRC5 cells.

The results presented in Figure 7 revealed that the purine-rich sequence is involved in the inclusion of the EDIIIA exon in both WI38VA13 and MRC5 cells since the deletion leads to the loss of inclusion. This loss of inclusion not only shows the importance of the purine-rich sequence for the inclusion of EDIIIA but also supports a possible role for the splicing enhancer in regulating the inclusion of the EDIIIA exon. Because loss of inclusion was complete in both cell lines, it was not possible to firmly conclude that the splicing enhancer plays a role in regulation. However, because the level of inclusion product is, as far as we can tell, equal in MRC5 and WI38VA13 cells (near 0%), we can argue that the splicing enhancer is possibly involved in regulating the inclusion of EDIIIA. The fact that the change in the amount of inclusion product was more drastic in WI38VA13 than in MRC5 cells is consistent with a function in regulation. However, we cannot rule out the possibility that the level of amplified inclusion products are not equivalent between WI38VA13 and MRC5, because the RT-PCR technique may be unable to distinguish between very small differences.

Our interpretation that the splicing enhancer is involved in regulation is supported by the following observations. First, SR proteins have been shown to bind to splicing enhancer (Lavigueur *et al.*, 1993). Second, the relative concentration of individual SR proteins varies between MRC5 and WI38VA13 cells (Fig. 8 and Chabot *et al.*, 1992). Thus, if SC35, SF2, or SRp40 binds to the splicing enhancer, their reduction in MRC5 cells would suggest that the SR/enhancer interaction may determine the level of EDIIIA inclusion.

This interpretation would be validated if we could show that the SR protein that actually binds to the splicing enhancer is differently expressed between cells that spliced fibronectin differently. To further address the role of the purine-rich sequence in the regulation of the alternative splicing of the EDIIIA exon, we relied on the expression of SR proteins *in vivo*. If the amount of one of the SR proteins is involved in the regulation of alternative splicing of EDIIIA, an overexpression of this SR protein in MRC5 cells would be predicted to increase the level of EDIIIA inclusion. If it were not involved in the regulation, little change in the level of the EDIIIA exon inclusion would be noted and/or a similar change would be expected if this protein was overexpressed in WI38VA13 and MRC5 cells.

To determine if any of the three SR proteins SC35, SF2, or SRp40 had an effect on the splicing pattern of the EDIIIA exon, we attempted to overexpress each of these SR protein in MRC5 cells (which are expressed in lower amounts than in WI38VA13 cells). Following selection of stable transformants, we examined the endogenous fibronectin splicing pattern. The results obtained did not allow any conclusion to be reached on the role of SF2 or SC35 since the transfected cells did not grow. A possible reason for this inhibition may be related to the importance of these SR proteins in constitutive splicing. However, a stable cell line was obtained following transfection with the SRp40 cDNA. Surprisingly, this cell line displayed a decrease in the amount of phosphorylated SRp40. This decrease was accompanied by a reduction in the level of EDIIIA inclusion product, suggesting a possible role for this SR protein in regulating the inclusion of the EDIIIA exon. The reason for this decrease in the level of SRp40 is unknown. However, it is possible that there is overexpression of non-phosphorylated SRp40 (which would not be

detected by the mAb104 antibody). This overexpression may lead to an unbalance in the cell (for example by sequestering a kinase), ultimately leading to a reduction in the level of phosphorylated SRp40. Because phosphorylated SR proteins are thought to represent the active forms of these proteins, a decrease in the level of phosphorylated SRp40 may be responsible for the effect on EDIIIA inclusion. This possibility would need to be confirmed.

Splicing enhancers have revealed themselves as very important, contributing to splice sites recognition in both constitutive and alternative splicing (Black, 1995; Chabot, 1996). These sequences act as stimulators by helping the recognition of weak 3' splice sites (Lavigueur *et al.*, 1993; Sun *et al.*, 1993a, 1993b). Known splicing enhancers are purine-rich and are bound by SR proteins. It is believed that the binding of the SR proteins to this sequence stimulates the binding of $U2AF^{65}$ to the pyrimidine track of the 3' splice site through the recruitment of $U2AF^{35}$ (Wang *et al.*, 1995; Zuo and Maniatis, 1996).

Studies to determine if the SR proteins could bind to the same purine-rich sequences revealed that they had distinct abilities to bind specific sequences (Tacke and Manley, 1995). This observation combined with the distinct tissue-specific distribution of SR proteins allows for the possibility of a regulatory system for splicing where different SR proteins would bind to specific sequences and influence the tissue-specific splicing of exons. Regulation would then arise from variations in SR proteins between different cell types and during the different stages of development. However, there is as yet no direct evidence that the SR protein/enhancer interaction is implicated in regulation of splicing in mammalian cells. Work on the regulation of the alternative splicing of exon 5 of the

cardiac troponin T gene revealed that its splicing enhancer sequence was not involved in the regulation of splicing but was needed for the inclusion of the exon in the mRNA (Xu *et al.*, 1993). Whether this is a general feature of splicing enhancers or is due to the small size of the cardiac troponin T exon 5 must await further investigations.

Evidence that a splicing enhancer is involved in the regulation of alternative splicing came from the studies done on the doublesex (dsx) gene involved in the sex determination of Drosophila (Hedley and Maniatis, 1991; Hoshijina *et* al., 1991; Tian and Maniatis, 1992). Regulation in this system involves the binding of the Tra and Tra-2 proteins to a splicing enhancer element located more than 300 nt downstream from the female-specific 3' splice site. SR proteins also bind to a purine-rich sequence located within this splicing enhancer. The SR proteins were found not to be directly involved in the regulation of alternative splicing of this exon, they were proposed to be important for mediating the interactions between splicing regulators (Tra and Tra-2) and the general splicing factor $U2AF^{65}$ (Tian and Maniatis, 1994; Zuo and Maniatis, 1996). With these results, Tian and Maniatis (1994) proposed that there is two types of splicing enhancers, one involved in regulation of sex determination to which sex-specific proteins bind, and constitutive splicing enhancers containing purine-rich sequences bound by SR proteins. Thus, the regulation of splicing of Drosophila doublesex pre-mRNA appears to be mediated by the regulator proteins Tra and Tra-2 and is aided by the SR proteins. If the SR proteins are important in the alternative splicing of the doublesex gene but regulation is actually achieved by the Tra and Tra-2 regulatory proteins we could consider this scenario as one way of regulating alternative splicing. This does not mean that all regulated cases of alternative splicing follow a similar path. A second regulatory system

could involve only the SR proteins as regulatory factors if the expression of SR proteins is tissue-, or cell-specific.

In the case of the fibronectin EDIIIA exon, no regulatory factor has yet been identified. However, we know that the purine-rich sequence is important for both *in vivo* and *in vitro* splicing of this exon, and that cell-specific splicing differences are eliminated by deleting the purine-rich element. We also know that the SR proteins bind to the purine-rich sequence of EDIIIA and that the distribution of SR proteins varies in cells that splice EDIIIA differently. These results suggest that the SR proteins may be involved in regulating exon EDIIIA splicing.

The identity of the SR proteins involved remains puzzling. A similar purine-rich sequence as that found in exon EDIIIA has been identified in the human growth hormone exon 5. This purine-rich element was shown to interact with SF2 but not SC35. Although SF2 and SC35 levels are lower in MRC5 cells, which exclude EDIIIA more frequently than WI38VA13 cells (Fig. 7), we were unable to verify the contribution of these two SR proteins to EDIIIA splicing because stable transformants were growth deficient. Interestingly, Taub *et al.* (1993) have shown that SRp40/HRS is expressed at a higher level in regenerating adult rat liver which includes fibronectin EDIIIA, in contrast to normal adult liver. We noted a difference in the level of expression of SRp40 between WI38VA13 and MRC5 cells (Fig. 8). Moreover, the decrease in the amount of SRp40 in MRC5 cells was associated with a decrease in EDIIIA inclusion, consistent with a role for SRp40 in regulating exon EDIIIA splicing.

Our studies have focused on the purine-rich sequence of the EDIIIA exon and did not consider the possible importance of two other elements which may be implicated in

the alternative splicing of this exon. First, there is the 5 nt sequence identified by Caputi *et al.* (1993), proposed to have a negative effect on EDIIIA exon splicing. The second is another purine-rich sequence in exon +1, similar to the one present in the EDIIIA exon (GGAGAAGAC). The importance of these sequences in the regulation of alternative splicing, if any, has yet to be determined. However, the possibility that regulation of the alternative splicing of the EDIIIA exon involves the purine-rich sequence and SRp40 is supported by the results presented in this study.

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